PATENT
Docket No. 275.00030102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	LI et al.	)	Group Art Unit: 1635
Serial No.:	10/038,984	)	Examiner: Tracy Ann Vivlemore
Filed:	January 4, 2002	)	Confirmation No. 9705

For: COMPOSITION AND METHOD FOR *IN VIVO* AND *IN VITRO* ATTENUATION OF GENE EXPRESSION USING DOUBLE STRANDED RNA

DECLARATION UNDER 37 C.F.R. §1.132 OF YIN-XIONG LI

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1. I, Yin-Xiong Li, declare and say as follows:

2. I received an M.D. with high honors from Hunan Medical University, China, in 1985 and received a Ph.D. in Molecular Biology and Biochemistry from Peking Union Medical College in 1991. In 1992 I was awarded the NIH Fogarty International Young Scientist Fellowship from the U.S. Department of Health and Human Services, NIH, and came to the United States to pursue research focused on an antisense RNA approach to knockdown wnt-1 oncogene expression and reverse malignant phenotype of mammary tumor cells. I was a research associate in the Developmental Biology Program, Institute of Molecular Medicine and Genetics at the Medical College of Georgia, from 1993 to 2001, and an Assistant Professor in the Departments of Pediatrics, Cell Biology and Medicine at Duke University Medical Center from 2001 to 2010. I am an author of over 28 published papers and inventor of an issued patent related to my research.

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2. I am a co-inventor of the subject matter claimed in the above identified patent application and have read and am familiar with its contents. I have also read and am familiar with the Office Action mailed August 19, 2009. I make this Declaration in support of the patentability of the pending claims.

3. The Examiner asserts that Zhao et al. (Developmental Biology, 2001, 229:215-223) report that injection of dsRNA resulted in degradation of endogenous mRNA and has a nonspecific effect at the posttranscriptional level, and that Oates et al. (Developmental Biology, 2000, 224:20-28) teach that dsRNA injected into early zebrafish embryos produced a nonspecific depletion of several endogenous mRNAs. It is my position that one cannot conclude from Zhao et al. that injection of dsRNA into zebrafish embryos causes degradation of endogenous mRNA and a nonspecific effect at the posttranscriptional level. It is also my position that one cannot conclude from Oates et al. that injection of dsRNA into early zebrafish embryos causes a nonspecific depletion of several endogenous mRNAs.

4. Injection of zebrafish embryos is routine; however, it requires much practice before it can be used successfully. Several aspects can lead to poor reproducibility, including the easily damaged delicate structure of a zebrafish egg, the fast development of the embryo after fertilization, and human fatigue. Failure to correctly introduce RNAs into zebrafish embryos can result in dramatic physical damage to the embryo. The types of phenotypes observed by Zhao et al. and Oates et al. can be explained as the result of physical damage to the embryo during injection.

5. In Oates et al., injections of dsRNA often resulted in non-specific phenotypes such as cyclopia, loss of notochord, and reduced brain structures that indicate that there is physical damage in early stages. This interpretation is further supported by the observation in Figure 3, in which the defects are asymmetrically distributed in one side of the embryo, such as the asymmetric eye and brain illustrated in figure 3k and 3l. These defects often occur when the injection is performed at the two cells stage and the injection is only done in one of the two cells

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with physical damage. Consequently, this type of damage causes typical defects that are only located on one side, but not the other side of the fish at late stages. Oates et al. specifically teach and use a method that one cell of zebrafish embryos at the two cell stage were injected with RNA.

6. Zhao et al. reported similar abnormalities with more severe deformities, including defective head, short tail, distorted midline structures, and abnormal somites. Evidence from Zhao et al. supports the position that the embryos are being physically damaged during the injection of dsRNA.

The embryos of figure 3b (injected with dsRNA), and figure 3d (also injected with dsRNA) show detachment of cells from the top of the embryo which leads to a cloudy substance flowing out of the side of the embryo. Figure 3b also shows leakage of yolk at the bottom of the yolk sack, which appears to be the result of piercing of the bottom of the yolk sack with the injection needle. The embryos of figures 3b and 3d also show leakage of cytoplasm from the top of the embryo. The embryos of 3f and 3g show severe physical yolk damage on one side (3f) or both sides (3g) of the embryos, and these embryos could not recover from the physical damage within 20 hours after the injections. The embryos shown in 3h are basically deformed, all having a very odd shape of the embryonic yolk that indicates severe damage resulting from injections. However, in figure 3 the embryos in control groups (3a, 3c and 3e), all show no signs of physical damage or are fully recovered at all stages of development.

In figure 5c of Zhao et al. the embryo has a bulge, and this is due to physical damage which may have resulted during injection of the embryo. Figure 7c (embryos injected with GFP mRNA and pouII-1 dsRNA), figure 7e (embryos injected with GFP dsRNA) and figure 7f (embryos injected with pouII-1 dsRNA) show embryos with more damage in comparison to the control groups in figure 7a (injected with GFP mRNA) and figure 7d (injected with pouII-1 sense RNA). Severely damaged embryos should be identified and removed from a collection of injected cells at the time point of 3-5 hours after the injections, since the damaged embryos and their degradation products can influence and damage other embryos in the same solution. This effect contributes to non-specific phenotypic changes during development.

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The double injection method used in Zhao et al. may have further contributed to the severe damage observationed. The protocol of Zhao et al. instructs the injection of GFP mRNA and dsRNA into a 1-2 cell stage embryo using two separate injections. This technique is unlikely to be successful, since pushing the needle against the cell wall to inject the second RNA will result in leakage of cytoplasm and yolk from the embryo through the hole generated from the first injection.

7. The phenotypes observed by Zhao et al. and Oates et al. can also be explained by the use of organic solvents in the preparation of the dsRNAs injected into embryos. Both Zhao et al. and Oates et al. use dsRNA that has been purified by phenol/chloroform extraction. Phenol is toxic to zebrafish embryos and causes concentration dependent adverse effects in zebrafish. Thus, the non-specific effects observed by Oates et al. and Zhao et al. are thought to be the result of toxic effects from residual phenol and/or chloroform in the dsRNA mixture injected into the embryos.

8. Zhao et al. also teach that the sense and antisense RNA is annealed in 1.5 M sodium chloride and 0.667 M sodium bicarbonate. The use of sodium increases chance of contaminating an embryo with sodium, and it is well known that sodium in injection solution is toxic for zebrafish embryonic development. The mixed sense and antisense RNA is incubated at 100°C for 10 minutes, then incubated at 55°C for 2 hours, and finally incubated at 37°C overnight. After annealing, RNA is treated with RNase One. This procedure may cause random degradation of the RNAs. These methods could result in the non-specific effects observed by Zhao et al.

9. I respectfully submit that the evidence above establishes that Zhao et al. and Oates et al. cannot be relied upon in demonstrating that injection of dsRNA causes degradation of endogenous non-target mRNA and nonspecific effects in zebrafish embryos.

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10. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Tile 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent that issues thereon.

Date: 7/18/2010 Signed: Yin-Xiong Li

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